

Published in final edited form as:

*Mol Microbiol.* 2007 September ; 65(5): 1300–1310. doi:10.1111/j.1365-2958.2007.05868.x.

## Polyhead formation in phage P22 pinpoints a region in coat protein required for conformational switching

Kristin N. Parent<sup>†</sup>, Margaret M. Suhanovsky<sup>†</sup>, and Carolyn M. Teschke<sup>\*</sup>

Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT, USA

### Summary

Eighteen single amino acid substitutions in phage P22 coat protein cause temperature-sensitive folding defects (*tsf*). Three intragenic global suppressor (*su*) substitutions (D163G, T166I and F170L), localized to a flexible loop, rescue the folding of several *tsf* coat proteins. Here we investigate the *su* substitutions in the absence of the original *tsf* substitutions. None of the *su* variant coat proteins displayed protein folding defects. Individual *su* substitutions had little effect on phage production *in vivo*; yet double and triple combinations resulted in a cold-sensitive (*cs*) phenotype, consistent with a defect in assembly. During virus assembly and maturation, conformational switching of capsid subunits is required when chemically identical capsid subunits form an icosahedron. Analysis of double- and triple-*su* phage-infected cell lysates by negative-stain electron microscopy reveals an increase in aberrant structures at the *cs* temperature. *In vitro* assembly of F170L coat protein causes production of polyheads, never seen before in phage P22. Purified procapsids composed of all of the *su* coat proteins showed defects in expansion, which mimics maturation *in vitro*. Our results suggest that a previously identified surface-exposed loop in coat protein is critical in conformational switching of subunits during both procapsid assembly and maturation.

### Introduction

A single amino acid substitution can disrupt protein folding, resulting in aggregation (Thomas *et al.*, 1995). Second-site amino acid substitutions, termed suppressor substitutions (*su*), have been isolated that are able to alleviate the misfolding caused by the original *tsf* substitution (Mitraki *et al.*, 1991; Beißinger *et al.*, 1995; Sideraki *et al.*, 2001). Sites of both the single amino acid substitutions and second-site suppressor substitutions pinpoint areas in the protein that are crucial for the proper folding and stability of the molecule. Here, we investigate the role of the *su* substitutions on the folding and assembly of a viral capsid protein in the absence of the original substitution.

Bacteriophage P22, a double-stranded (ds) DNA phage that infects *Salmonella enterica* serovar Typhimurium, has been studied extensively as a model for both virus assembly and protein folding (Casjens, 1979; Fuller and King, 1980; Fuller and King, 1982; Casjens *et al.*, 1985; Prevelige *et al.*, 1993; Teschke and King, 1993; 1995; Parent *et al.*, 2006). Viral capsids are not immobile static structures, but instead are large macromolecular assemblies that undergo significant conformational changes as part of their normal morphogenesis (Caspar, 1980; Steven *et al.*, 1997; Steven *et al.*, 2005). The bacteriophage P22 capsid lattice is composed of solely the coat protein, and all copies of the coat protein are chemically identical with no post-translational modifications (Casjens and King, 1974). According to

© 2007 The Authors

<sup>\*</sup>For correspondence. teschke@uconn.edu; Tel. (+1) 860 486 4282; Fax (+1) 860 486 4331.

<sup>†</sup>These authors contributed equally to this manuscript.

the theory of quasi-equivalence, these coat proteins must be plastic enough to adopt several different conformations during assembly, a process known as conformational switching. During capsid maturation, these subunits undergo additional conformational changes (Caspar and Klug, 1962; Prasad *et al.*, 1993; Johnson and Speir, 1997).

During phage P22 assembly, 415 coat protein monomers co-assemble with 60–300 scaffolding protein molecules (Casjens and King, 1974; Casjens, 1979; Eppler *et al.*, 1991; Thuman-Commike *et al.*, 1996; Parent *et al.*, 2006) into a precursor structure termed the procapsid. In addition, 12–20 copies of each of the injection proteins, gp7, gp16 and gp20 (Botstein *et al.*, 1973; Israel, 1977), and the unique portal complex (Bazinet *et al.*, 1988), are incorporated into procapsids. Upon completion of the procapsid, dsDNA is packaged via the portal protein complex (Casjens and King, 1974), and the scaffolding protein exits, likely through holes in the procapsid lattice (Earnshaw *et al.*, 1976; Prasad *et al.*, 1993). Concomitantly, a change in overall capsid morphology during capsid maturation converts the round-appearing procapsid to the classic icosahedral shape of many viruses (Prasad *et al.*, 1993), and is accompanied by an expansion in head volume by ~10% (Earnshaw *et al.*, 1976; Prasad *et al.*, 1993; Teschke *et al.*, 2003). Finally, the phage head is stabilized by the addition of plug and tailspike proteins to form the mature, infectious phage (Strauss and King, 1984).

The folding of phage P22 coat protein has been extensively studied using classic mutational analysis. Eighteen single amino acid substitutions that render the molecule temperature-sensitive for folding (*tsf*) have been characterized (Gordon and King, 1993; Teschke and King, 1995). In addition, several second-site suppressors have been found that alleviate these folding defects (Aramli and Teschke, 1999). Three of these *su* substitutions are ‘global’ suppressors, D163G, T166I and F170L, and alleviate folding defects from more than one original substitution. Although a high-resolution structure is not available for P22 coat protein, two putative folding domains have been proposed to be joined by a flexible hinge region comprising residues 157–207 (Lanman *et al.*, 1999; Kang *et al.*, 2006). The three global suppressors lie within the hinge loop and are able to rescue *tsf* substitutions that are contained in either domain.

We have shown that *tsf*:T166I and *tsf*:F170L proteins function differently to alleviate folding defects. *Tsf*:T166I coat proteins induce and recruit the molecular chaperone complex GroEL/S by increasing the population of folding intermediates. The resulting increase in native monomer concentrations drives procapsid formation (Aramli and Teschke, 2001; Parent *et al.*, 2004). Conversely, *tsf*:F170L coat proteins stabilize the native state of the protein to alleviate aggregation (Parent and Teschke, 2007). The *tsf*:D163G coat proteins demonstrated folding and assembly behaviour similar to those containing *tsf*:T166I coat protein (Parent and Teschke, 2007).

Here, we investigated the effect of the global *su* substitutions in the absence of the original *tsf* substitutions. We present evidence that combinations of the *su* substitutions negatively affect phage production; specifically, they affect the ability of the coat protein to undergo the conformational switching necessary to promote proper procapsid assembly and capsid maturation.

## Results

### Global suppressors demonstrate a *cs* phenotype *in vivo*

Plating experiments that count the number of phage produced from infected cells are a convenient way to determine whether variants of coat protein can fold under different conditions, as misfolded coat proteins cannot assemble into infectious phage. Phage

containing an amber mutation in gene 5, which codes for coat protein, were plated on *Salmonella* cells containing a plasmid that encoded coat protein, either wild-type (WT), the individual *su* substitutions, or the double and triple combinations of the *su* substitutions as listed in Table 1. In this experiment, phage are only produced by complementation through expression of coat protein from the plasmid. The phage were plated at temperatures ranging from 16°C to 41°C on cells that expressed WT coat protein and each coat variant. The relative titre is the titre at each temperature divided by the titre of phage produced for each strain when plated under the permissive condition, 30°C. Phage production with WT coat protein or the individual *su* coat proteins, D163G, T166I and F170L, was unaffected by temperature, indicating effective protein folding at the various temperatures (Fig. 1A). The small decrease in relative titre at 16°C is due to an inefficiency in complementation at the low temperature. However, expression of the double and triple combinations of *su* substitutions, D163G:T166I, D163G:F170L, T166I:F170L and D163G:T166I:F170L, caused a strong *cs* phenotype (Fig. 1B). Expression of any of the three double-*su*-substituted coat proteins (D163G:T166I, D163G:F170L, T166I:F170L) resulted in a *cs* phenotype at 16°C. Phage complemented by the triple-*su*-substituted coat protein, D163G:T166I:F170L, show a *cs* phenotype at 25°C.

We tested the ability of the purified single-, double- and triple-*su* coat proteins to fold properly *in vitro* through use of several of our standard protocols (Teschke and King, 1993; 1995; Teschke and Fong, 1996; Fong *et al.*, 1997; Teschke, 1999; Aramli and Teschke, 2001). In all cases, the *su* variant coat proteins behaved similarly to WT, indicating that the *cs* phenotype observed in Fig. 1 was not due to a protein folding defect (data not shown). The extreme *cs* phenotype of the triple *su* variant, where phage production is inhibited at 25°C, has not yet been reported for any phage P22 coat protein variants and is likely due to a more severe assembly defect than has been characterized previously (Teschke and Fong, 1996).

### ***Su* coat proteins result in increased empty-head formation**

Phage P22 coat protein variants with a *cs* phenotype could be indicative of assembly defects as reported for the coat variants T10I, R101C and N414S (Teschke and Fong, 1996). Even during a normal infection with WT phage, a very small fraction of particles demonstrate aborted assembly, and result in non-infectious empty heads, which are marked by a lack of scaffolding protein or DNA (Lenk *et al.*, 1975). Empty heads can be easily separated from procapsids and mature phage by sucrose gradient sedimentation (King *et al.*, 1973). To discern whether an increase in empty heads could be responsible for the *cs* phenotype of the double and triple combinations of *su* coat proteins, we separated the products from a 16°C infection by sedimentation through linear 5–20% sucrose gradients and compared the fraction of empty heads from the *su* coat proteins with WT coat protein. In addition, negative-stain electron microscopy (EM) was used to visualize the products of the assembly reactions.

Figure 2 shows the sucrose gradient density profiles of 16°C cell lysates from phage-infected cells expressing WT and *su* coat proteins. Sucrose gradient fractions were analysed by SDS-PAGE and densitometry as described previously (Parent *et al.*, 2007). The fraction of coat protein shown in the profiles was determined by dividing the intensity of coat protein in each fraction by the total density of coat protein bands. For all infections with variants and WT, the total coat protein expression levels and the total coat protein able to assemble into complexes were similar (data not shown). However, the distribution of coat protein found as procapsids, phage and aberrant structures was different for each protein type.

When WT coat protein was expressed at 16°C, the phage infection resulted in two distinct peaks on the sucrose gradients: normal procapsids (Fig. 2, peak 2) and mature phage

particles (Fig. 2, peak 3), with minimal aberrant structures co-sedimenting with the mature phage (Fig. 2, peak 3), consistent with previous reports (Lenk *et al.*, 1975). When the *su*-substituted coat proteins were expressed in single, double and triple combinations, an additional peak was observed (Fig. 2A, peak 1). Micrographs of negatively stained samples from peak 1 are shown in Fig. 3A. The resulting particles appear similar to the empty heads reported previously (Lenk *et al.*, 1975). As a control we ran a sample of purified empty procapsid shells (procapsids stripped of scaffolding protein) on a sucrose gradient. The purified shells migrated in approximately the same position as empty heads and are shown in Fig. 3B.

Surprisingly, a large peak at the bottom of the gradient was observed even for variants that show a strong *cs* phenotype (Fig. 2, peak 3). Based on the data presented in Fig. 1, it was unlikely that large quantities of mature phage were being produced at 16°C with the double- and triple-*su* coat proteins. Micrographs of negatively stained samples from representative proteins (WT, F170L, T166I:F170L and D163G:T166I:F170L) showed that in the gradients from infections expressing the double- or triple-*su* coat proteins, peak 3 comprised mostly large spiral and irregular structures (Fig. 3A, peak 3). In contrast, in infections expressing WT coat protein mature phage were present along with a small amount of aberrant forms, consistent with our plating experiments shown in Fig. 1. In all cases, peak 2 corresponded to normal-appearing procapsids, containing the appropriate complement of proteins (data not shown), indicating that WT, as well as all the *su* variants observed, are competent for procapsid assembly.

To determine the percent of aberrant structures formed with the variant and WT coat proteins shown in Fig. 3A, we quantified the number of particles from multiple micrographs. We analysed between 600 and 900 particles for each protein and normalized the data based on the sucrose gradient profiles. We considered empty heads, spirals and capsids with incorrect geometry to be 'aberrant' structures (Fig. 3C). The lowest percentage of incorrect particles was observed with WT coat protein. The single- and double-*su* coat proteins had slightly more aberrant structures compared with WT coat protein. The triple-*su* coat protein had significantly more aberrant structures than any other protein type (Fig. 3C). Representative samples are shown; the same trend was observed for all single and double combinations of *su* substitutions. Our data indicate that the *su* substitutions affect the ability of coat protein to assemble correctly *in vivo*.

### Spirals and aberrant structures are prevalent *in su* assembly reactions *in vitro*

The *in vitro* assembly of the single-, double- and triple-*su* coat proteins was analysed to determine the effect of the *su* substitutions in the absence of other capsid proteins, aside from scaffolding protein. The single-, double- and triple-*su* coat proteins were purified, as described previously (Fuller and King, 1981; Teschke and King, 1993; Parent *et al.*, 2005). A series of *in vitro* assembly reactions were performed, where WT or each variant coat protein was mixed with scaffolding protein, and the products were visualized by negative-stain EM (Parent *et al.*, 2005; Parent *et al.*, 2006). As reported previously, the resulting particles from *in vitro*-assembled WT coat protein were similar in size and shape to procapsids formed *in vivo* (Fuller and King, 1982; Prevelige *et al.*, 1988; Parent *et al.*, 2005) (Fig. 4). The *su* variants showed different degrees of assembly competence. The *su* coat proteins D163G and T166I resulted in formation of what appeared to be spirals, incomplete structures and petite capsids, as well as normal procapsids (Fig. 4). Tube-like structures appeared in the *in vitro* assembly reactions formed from F170L coat proteins (Fig. 4), and were similar to polyhead structures previously reported for polyoma virus (Baker and Caspar, 1983), bacteriophage T4 (Steven *et al.*, 1976), SPO1 (Parker *et al.*, 1983), cowpea chlorotic mottle virus (Bancroft *et al.*, 1976) and lambda phage (Georgopoulos *et al.*, 1973). For the double and triple combinations of the *su* coat proteins, altered products including

spirals and petite capsids were also present (Fig. 4). Our *in vitro* results are consistent with our *in vivo* experiments and suggest that the *su* substitutions affect conformational switching during procapsid assembly.

### Procapsids formed from *su* coat proteins have altered ability to expand

As the *su* coat proteins can assemble into a significant population of correctly sized procapsids both *in vivo* and *in vitro*, we investigated why procapsids are unable to produce active phage. Because capsid subunit plasticity is observed not only during formation of the procapsids but also during the maturation event, we tested the ability of procapsids formed from the variant proteins to undergo the maturation step. This step involves a large conformation change and results in expansion in volume. Expansion of procapsids can be induced *in vitro* by addition of chemical or heat treatment (Galisteo and King, 1993). The presence of procapsids and resulting expanded heads can be observed by agarose gel electrophoresis because expanded heads migrate more slowly than procapsids (Teschke *et al.*, 2003).

We tested the ability of the variant procapsids to expand due to exposure to low pH. The particles in the reaction were visualized on agarose gels. For all variants, the procapsids remained unchanged in buffers that ranged between pH 7.6 and pH 3.5 (data not shown). Figure 5 shows the products of the reaction when procapsids composed of WT, single, double and triple combinations of the *su* substitutions were incubated at pH 3.0. WT procapsids expanded fully in less than 1 h. The procapsids composed of the single-*su* variations, D163G and T166I, expanded to forms that run on the agarose gel in a position similar to WT expanded heads, although at a shorter incubation time for D163G, but at a longer incubation for T166I. For F170L, expanded heads could be seen at 120 min, but by 240 min, all of the protein is monomeric.

For the double- and triple-*su*-substituted coat proteins, expansion did not readily occur. Although procapsids generated from the D163G:T166I variant demonstrate some ability to expand, much of the intensity is lost to monomer species at longer times. For procapsids composed of T166I:F170L or D163G:T166I:F170L coat protein, expansion did not occur. Interestingly, D163G:F170L did not seem able to expand in the same cooperative manner as WT, but instead showed bands with distinct migration seen at the time points of 20 and 30 min, possibly reflecting expansion intermediates. These data indicate that the double and triple combinations of the *su* variant coat proteins were incompetent to undergo proper expansion *in vitro*.

In order to confirm the data presented in Fig. 5, we performed *in vitro* maturation reactions of the purified procapsids using heat rather than pH. Procapsids were incubated at 71°C for times ranging from 0 to 250 min. The fraction of procapsids remaining was determined by densitometry (Fig. 6). Consistent with the data presented above, procapsids composed of single-*su* substitutions, and the double and triple combinations, required much longer times at elevated temperature to induce the conformational transition as compared with WT coat protein. WT procapsids expanded rapidly, with complete procapsid loss in less than 15 min. In addition, the *su* procapsids demonstrated a biphasic transition during the transition. Our data are consistent with data from the Prevelige laboratory, where other variants within the hinge loop also showed biphasic kinetics of expansion. In contrast to our results, these variants increased the rate of expansion relative to WT coat protein (Kang *et al.*, 2006). Nonetheless, the combined data highlight the importance of this region in the maturation reaction.

Lastly, the stability of shells and expanded heads formed by the single-, double- and triple-*su* coat proteins was compared with WT coat protein by titrations with the denaturant

guanidine hydrochloride, as described previously (Capen and Teschke, 2000; Parent *et al.*, 2007). The stability of the shells was not significantly different (data not shown). The expanded heads also did not show changes in stability. Therefore, the change in incubation time required to expand the shells by either pH or heat is likely due to an increase in activation energy for the maturation transition, rather than a change in stability of the particles. In total, these data suggest that the *su* substitutions affect conformational switching during both pro-capsid assembly and maturation.

## Discussion

All icosahedral viruses share the problem of organizing the capsid protein subunits into capsomers that promote formation of a closed sphere. For viruses such as bacteriophage P22, the capsid lattice is formed by pentons with fivefold symmetry at the 12 vertices and hexons with sixfold symmetry that make up the  $T = 7$  geometry of the 20 facets. For P22, the capsomers consist of the same type of subunit; all 415 copies are chemically identical copies of coat protein. Nevertheless, P22 procapsid assembly occurs through addition of monomeric coat subunits to the growing edge of the procapsid (Prevelige *et al.*, 1993). One fundamental, yet unanswered, question is how viruses accomplish the task of assembly with such high fidelity.

Here we have presented data that indicate that new variations within the flexible hinge region in P22 coat protein affect the ability of coat protein to assemble into the proper lattice. Interestingly, these substitutions were isolated to alleviate folding defects of amino acid substitutions that cause a temperature-sensitive phenotype; yet when expressed without the *tsf* substitutions, the *su* coat proteins do not demonstrate any effects on protein folding. The effects on assembly of the individual *su* substitutions are rather mild, with only the triple combination showing a severe phenotype, illustrating that the phage are able to tolerate a fair amount of sequence variation and still function properly. The effect of each substitution is specific; two substitutions (D163G and T166I) promote spiral formation as well as the formation of capsids with incorrect T-number. One substitution (F170L) promotes polyhead formation. The effect of the substitutions is additive *in vivo*; when combinations of two or three substitutions are present, phage growth becomes cold-sensitive, a phenotype that is a hallmark of assembly defects (Teschke and Fong, 1996). We propose that the individual *su* substitutions highlight the hinge region of P22 coat protein as crucial for the proper subunit conformational switching required for quasi-equivalence during assembly of P22 procapsids. Our data suggest that, by promoting an imbalance of conformations required to generate hexons or pentons during assembly, the procapsids are not able to achieve the normal  $T = 7$  capsid lattice.

The *su* substitutions also affect the ability of the pro-capsid to undergo the lattice transition required for maturation. Conformational flexibility is also a necessary factor during the expansion and maturation process for many tailed bacteriophage and animal viruses (Caspar and Klug, 1962; Johnson and Speir, 1997). During this process, round-appearing procapsids must undergo a flattening of their sides and an outward movement of the pentons to form infectious virions. The final expanded heads generally have thinner capsid walls compared with the precursor procapsid, but are considerably more stable (Galisteo and King, 1993; Gan *et al.*, 2006; Lander *et al.*, 2006). These structural rearrangements are observed during P22 maturation, where a hinge region, comprising residues 157–207, becomes buried during the maturation transition (Lanman *et al.*, 1999; Kang *et al.*, 2006). The variants we present here are localized to the loop and affect expansion, again highlighting the importance of this region to maturation. Our data suggest that the activation energy barrier to expand is higher for the double- and triple-*su* variants. Indeed, so much energy appears to be required to traverse the activation energy barrier, the heads cannot remain intact, falling apart to inactive

subunits. Previously, we have shown through the use of electron cryo-microscopy that, during *in vitro* expansion of procapsids, the pentons are released (Teschke *et al.*, 2003). We hypothesize that by altering the *in vitro* expansion, the variations reported here change the coat protein subunit–subunit interactions, perhaps at the penton–hexon interface, leading to the increase in the activation energy barrier to expansion.

The hinge region for P22 encompasses the three global *su* substitutions that were isolated as second-site suppressors for protein folding defects in coat protein. Based on our data, we propose that the global *su* substitutions, when present in the absence of the original *tsf* substitution, alter the ability of the coat protein subunits to undergo correct conformational switching. In addition, we propose that conformational switching is affected not only during procapsid assembly, where spirals and polyheads are formed, but also in the maturation event, where correctly sized and organized procapsids have an increase in the activation energy barrier to expansion. Characterization of these variations has provided insight into how protein structure relates to function, how the dynamic properties of a complex protein machine are determined, and what variations are allowed in the evolution of a protein. Although a detailed structure of phage P22 could provide a more in-depth understanding of the above questions, as determination of the coat protein structure has not yet been possible, our work has defined amino acids critical for subunit–subunit interactions that regulate P22 virion formation.

## Experimental procedures

### Bacteria

*Salmonella enterica* serovar Typhimurium strain DB7136 (leuA414am, hisC525am, sup<sup>o</sup>) has been previously described in Winston *et al.*'s study (1979). The bacteria were transformed with a plasmid (pHBW1) that carried gene 5, which codes for P22 coat protein. The plasmid encoded WT coat protein or carried various mutations as listed in Table 1. To generate procapsids, cells expressing WT or *su* coat proteins (Table 1) from a plasmid were infected with phage that carried, in addition to the 5<sup>-</sup> amber allele, an amber in gene 2, which prevents DNA packaging, and an amber in gene 13 to prevent cell lysis.

### Construction of pHBW1

P22 DNA containing the coat protein gene (gene 5) was subcloned so that the P22 gene 5 was expressed from the *trc* promoter of the superlinker plasmid vector pSE380 (Invitrogen). The source of the P22 gene 5 insert was pCoat-1, generously donated by Sherwood Casjens. A Quikchange kit from Stratagene was used to alter pHBW1 to encode for the various mutations in coat protein. The primers used for the Quikchange reactions are listed in Table 1. The mutated plasmids were sequenced at the Yale Facility for DNA sequencing.

### Bacteriophage

The P22 bacteriophage used in these studies contained an amber (5<sup>-</sup> am N115) in gene 5. The amber was complemented by expression of coat protein from the various plasmids listed in Table 1. The P22 strain carried the c1–7 allele to prevent lysogeny. Bacteriophage preparation of the WT 5<sup>-</sup>amber strain was performed as previously described (Aramli and Teschke, 1999).

### Media

Luria broth (LB, from Invitrogen Life Technologies) was used to support bacterial growth for plating experiments, and for the preparation of phage stocks. The phage stocks were stored in M9 salts with 2 mM MgSO<sub>4</sub>. For all infections involving complementation of the

coat protein variants from a plasmid, the media were supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin and 2 mM IPTG to induce expression of coat protein.

### Relative titre determination

Phage contained an amber mutation in gene 5. Phage were plated on *Salmonella* DB7136 transformed with the plasmids described in Table 1 in the presence of 2 mM IPTG at temperatures from 16°C to 41°C. The relative titre was calculated by dividing the titre of each phage at the experimental temperature by the titre of the phage at 30°C.

### Analysis of *in vivo* phage production

Overnight cultures of DB7136 transformed with the plasmids listed in Table 1 were grown in LB supplemented with 100  $\mu\text{g ml}^{-1}$  ampicillin. The overnight cultures were used to inoculate a culture in the same media, grown at 16°C. The cells were grown to a density of  $2 \times 10^8$  cells  $\text{ml}^{-1}$ , and induced with 2 mM IPTG for 10 min prior to infection. Gene 5 amber phage at a multiplicity of infection of five were added to the cells, and the infected cultures were incubated overnight at 16°C.

The phage-infected cells were spun at 13.2 K r.p.m., 4°C for 5 min in a microfuge. The pelleted cells were suspended in lysis buffer (0.1% Triton, 100  $\mu\text{g ml}^{-1}$  lysozyme, 50 mM Tris HCl, 25 mM NaCl, 7 mM EDTA, pH 7.6), and frozen at -20°C. Cells were thawed on ice and treated with 20 mM  $\text{MgSO}_4$ , 100  $\mu\text{g ml}^{-1}$  RNase and 100  $\mu\text{g ml}^{-1}$  DNase. Samples were then frozen and thawed two times to ensure complete lysis. Cell debris was removed by centrifugation in a microfuge for 5 min at 4°C. Assembled phage proteins were concentrated by centrifugation for 40 min at 147 000 g. The pellets were resuspended in 50 mM Tris HCl, 25 mM NaCl, 5 mM EDTA, pH 7.6.

A 200  $\mu\text{l}$  aliquot of the resuspended sample was then applied to 2.2 ml of 5–20% sucrose gradient and run in a RP55S rotor in a Sorvall RC M120EX centrifuge for 35 min at 35 000 r.p.m. at 20°C. The linear sucrose gradients were made using a Gradient Master Model 106 (Biocomp Instruments). The gradients were hand-fractionated from the top into 100  $\mu\text{l}$  aliquots. A portion of each gradient fraction was run on a 10% SDS-polyacrylamide gel, and the protein bands were quantified by densitometry using a Kodak Electrophoresis Documentation and Analysis System (EDAS), and the other portion was visualized by negative-stain EM.

### Negative-stain EM

A portion of the procapsids purified from phage-infected cells, as well as the *in vitro* assembly reactions described below, were used for negative-stain EM. Three microlitres of the sample was allowed to absorb to a carbon-coated grid for 1 min. Two to three drops of water were used to wash the grid. The sample was stained with two to three drops of 1% aqueous uranyl acetate for 30 s. The excess liquid was wicked off and the grid air-dried. The samples were viewed using a Philips Model 300 TEM at 80 kV, with a final magnification of 71 300 $\times$ .

### Purification of coat proteins

Wild-type as well as the *su* proteins were obtained from empty procapsid shells that had been prepared as previously described (Anderson and Teschke, 2003; Parent *et al.*, 2005), except that the variant coat protein was expressed from a plasmid complementing a WT 2-5-13- phage, to generate procapsids containing the appropriate coat protein. The amber in gene 2 prevents DNA packaging and the gene 13 amber prevents cell lysis. Empty procapsid shells were generated from the purified procapsids by incubation of the pro-capsids with 0.5

M guanidine hydrochloride, which extracts the scaffolding protein and the minor capsid proteins while leaving the coat lattice intact.

### Assembly of refolded coat proteins

Empty procapsid shells were unfolded at 2 mg ml<sup>-1</sup> in 6.75 M urea, 20 mM sodium phosphate buffer (pH 7.6) for 30 min at room temperature. Refolding was initiated by continuous-flow dialysis with 20 mM phosphate buffer (pH 7.6) at 4°C, at a rate of 0.45 ml min<sup>-1</sup>. Coat protein monomers were collected when urea was no longer detected by refractometry. Aggregates and other structures were removed by centrifugation at 175 000 g for 20 min at 4°C. Refolded coat protein was added to scaffolding protein for a final concentration of 0.5 mg ml<sup>-1</sup> each (~11 μM coat protein and ~15 μM scaffolding protein), in the presence of 60 mM NaCl.

### Procapsid pH expansions and agarose gel electrophoresis

Procapsids purified from phage-infected cells were diluted to 1 mg ml<sup>-1</sup> with 20 mM citric acid at pH 3.0 and incubated at 37°C. Samples were taken with time ranging from 0 to 240 min, and then transferred to ice. The pH was adjusted back to neutral with agarose gel sample buffer. 1.2% Seakem HGT agarose gels were prepared and run as previously described with about 5 μg of protein loaded in each lane (Serwer and Pichler, 1978; Tuma *et al.*, 1998; Lanman *et al.*, 1999; Teschke *et al.*, 2003). The gels were run at 17 V constant for ~24 h at 4°C. The gels were stained for 1 h with 10% acetic acid containing 0.03% Coomassie brilliant blue R-250 and 0.02% Coomassie brilliant blue G-250, then destained over 2–3 days with a solution of 10% acetic acid and 10% isopropyl alcohol.

### Heat expansion of shells

Shells at 1 mg ml<sup>-1</sup> were incubated at 71°C in a thermal cycler. Aliquots were taken with time ranging from 0 to 250 min and diluted with ice-cold agarose gel sample buffer. Approximately 5 μg of protein was loaded in each lane of a 1.2% Seakem HGT agarose gel, as described above. The bands were quantified using Kodak Digital Science EDAS.

### Acknowledgments

We thank Dr Marie Cantino for use of the University of Connecticut Electron Microscopy Center and her expert guidance in electron microscopy. We also thank Hilary B. Whitlatch for creation of the pHBW1 plasmid. This work was supported by PHS Grant GM076661 to C.M.T., a PHS fellowship GM073598 to K.N.P., and a University of Connecticut Research Foundation Grant.

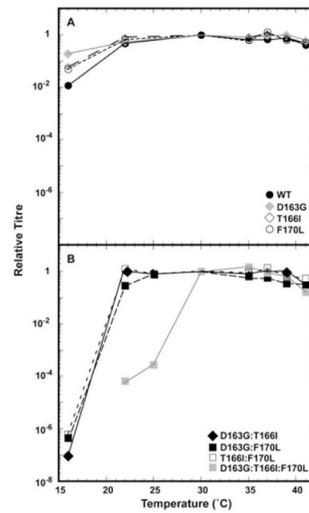
### References

- Anderson E, Teschke CM. Folding of phage P22 coat protein monomers: kinetic and thermodynamic properties. *Virology*. 2003; 313:184–197. [PubMed: 12951032]
- Aramli LA, Teschke CM. Single amino acid substitutions globally suppress the folding defects of temperature-sensitive folding mutants of phage P22 coat protein. *J Biol Chem*. 1999; 274:22217–22224. [PubMed: 10428787]
- Aramli L, Teschke C. Alleviation of a defect in protein folding by increasing the rate of subunit assembly. *J Biol Chem*. 2001; 276:25372–25377. [PubMed: 11304542]
- Baker TS, Caspar DLD. Polyoma virus ‘hexamer’ tubes consist of paired pentamers. *Nature*. 1983; 302:446–448. [PubMed: 6304526]
- Bancroft JB, McDonald JG, Rees MW. A mutant of cowpea chlorotic mottle virus with a perturbed assembly mechanism. *Virology*. 1976; 75:293–305. [PubMed: 12609]
- Bazinet C, Benbaset J, King J, Carazo JM, Carrascosa JL. Purification and organization of the gene 1 portal protein required for phage P22 DNA packaging. *Biochemistry*. 1988; 27:1849–1856. [PubMed: 3288279]

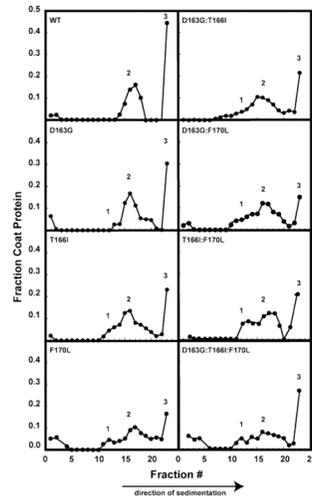
- Beißinger M, Lee SC, Steinbacher S, Reinemer P, Huber R, Yu MH, Seckler R. Mutations that stabilize folding intermediates of phage P22 tailspike protein: folding *in vivo* and *in vitro*, stability, and structural context. *J Mol Biol.* 1995; 249:185–194. [PubMed: 7776371]
- Botstein D, Waddell CH, King J. Mechanism of head assembly and DNA encapsulation in *Salmonella* phage P22. I. Genes, proteins, structures and DNA maturation. *J Mol Biol.* 1973; 80:669–695. [PubMed: 4773026]
- Capen CM, Teschke CM. Folding defects caused by single amino acid substitutions in a subunit are not alleviated by assembly. *Biochemistry.* 2000; 39:1142–1151. [PubMed: 10653661]
- Casjens S. Molecular organization of the bacteriophage P22 coat protein shell. *J Mol Biol.* 1979; 131:1–19. [PubMed: 385887]
- Casjens S, King J. P22 morphogenesis. I: catalytic scaffolding protein in capsid assembly. *J Supramol Struct.* 1974; 2:202–224. [PubMed: 4612247]
- Casjens S, Adams MB, Hall C, King J. Assembly-controlled autogenous modulation of bacteriophage P22 scaffolding protein gene expression. *J Virol.* 1985; 53:174–179. [PubMed: 3880825]
- Caspar DLD. Movement and self-control in protein assemblies: quasi-equivalence revisited. *Biophys J.* 1980; 32:103–138. [PubMed: 6894706]
- Caspar DLD, Klug A. Physical principles in the construction of regular viruses. *Cold Spring Harb Symp Quant Biol.* 1962; 27:1–24. [PubMed: 14019094]
- Earnshaw W, Casjens S, Harrison SC. Assembly of the head of bacteriophage P22: x-ray diffraction from heads, proheads and related structures. *J Mol Biol.* 1976; 104:387–410. [PubMed: 781287]
- Eppler K, Wykoff E, Goates J, Parr R, Casjens S. Nucleotide sequence of the bacteriophage P22 genes required for DNA packaging. *Virology.* 1991; 183:519–538. [PubMed: 1853558]
- Fong DG, Doyle SM, Teschke CM. The folded conformation of phage P22 coat protein is affected by amino acid substitutions that lead to a cold-sensitive phenotype. *Biochemistry.* 1997; 36:3971–3980. [PubMed: 9092827]
- Fuller MT, King J. Regulation of coat protein polymerization by the scaffolding protein of bacteriophage P22. *Biophys J.* 1980; 32:381–401. [PubMed: 7018607]
- Fuller MT, King J. Purification of the coat and scaffolding protein from procapsids of bacteriophage P22. *Virology.* 1981; 112:529–547. [PubMed: 7257185]
- Fuller MT, King J. Assembly *in vitro* of bacteriophage P22 procapsids from purified coat and scaffolding subunits. *J Mol Biol.* 1982; 156:633–665. [PubMed: 6750133]
- Galisteo ML, King J. Conformational transformations in the protein lattice of phage P22 procapsids. *Biophys J.* 1993; 65:227–235. [PubMed: 8369433]
- Gan L, Speir JA, Conway JF, Lander GC, Cheng N, Firek BA, et al. Capsid conformational sampling in HK97 maturation visualized by X-ray crystallography and cryo-EM. *Structure.* 2006; 14:1655–1665. [PubMed: 17098191]
- Georgopoulos CP, Hendrix RW, Casjens SR, Kaiser AD. Host participation in bacteriophage lambda head assembly. *J Mol Biol.* 1973; 76:45–60. [PubMed: 4578100]
- Gordon CL, King J. Temperature-sensitive mutations in the phage P22 coat protein which interfere with polypeptide chain folding. *J Biol Chem.* 1993; 268:9358–9368. [PubMed: 8486630]
- Israel V. E proteins of bacteriophage P22. I. Identification and ejection from wild-type and defective particles. *J Virol.* 1977; 23:91–97. [PubMed: 328927]
- Johnson JE, Speir JA. Quasi-equivalent viruses: a paradigm for protein assemblies. *J Mol Biol.* 1997; 269:665–675. [PubMed: 9223631]
- Kang S, Hawkrigde AM, Johnson KL, Muddiman DC, Prevelige PJ. Identification of subunit–subunit interactions in bacteriophage P22 procapsids by chemical cross-linking and mass-spectrometry. *J Proteome Res.* 2006; 5:370–377. [PubMed: 16457603]
- King J, Lenk EV, Botstein D. Mechanism of head assembly and DNA encapsulation in *Salmonella* phage P22. II. Morphogenetic pathway. *J Mol Biol.* 1973; 80:697–731. [PubMed: 4773027]
- Lander GC, Tang L, Casjens SR, Gilcrease EB, Prevelige PJ, Poliakov A, et al. The structure of an infectious P22 virion shows the signal for headful DNA packaging. *Science.* 2006; 312:1791–1795. [PubMed: 16709746]

- Lanman J, Tuma R, Prevelige PE Jr. Identification and characterization of the domain structure of bacteriophage P22 coat protein. *Biochemistry*. 1999; 38:14614–14623. [PubMed: 10545185]
- Lenk E, Casjens S, Weeks J, King J. Intracellular visualization of precursor capsids in phage P22 mutant infected cells. *Virology*. 1975; 68:182–199. [PubMed: 1103445]
- Mitraki A, Fane B, Haase-Pettingell C, Sturtevant J, King J. Global suppression of protein folding defects and inclusion body formation. *Science*. 1991; 253:54–58. [PubMed: 1648264]
- Parent KN, Teschke CM. GroEL/S substrate specificity based on substrate unfolding propensity. *Cell Stress Chaperones*. 2007; 12:20–32. [PubMed: 17441504]
- Parent KN, Ranaghan MJ, Teschke CM. A second site suppressor of a folding defect functions via interactions with a chaperone network to improve folding and assembly *in vivo*. *Mol Microbiol*. 2004; 54:1036–1054. [PubMed: 15522085]
- Parent KN, Doyle SM, Anderson E, Teschke CM. Electrostatic interactions govern both nucleation and elongation during phage P22 procapsid assembly. *Virology*. 2005; 340:33–45. [PubMed: 16045955]
- Parent KN, Zlotnick A, Teschke CM. Quantitative analysis of multi-component spherical virus assembly: scaffolding protein contributes to the global stability of phage P22 procapsids. *J Mol Biol*. 2006; 359:1097–1106. [PubMed: 16697406]
- Parent KN, Suhanovsky MM, Teschke CM. Phage P22 procapsids equilibrate with free coat protein subunits. *J Mol Biol*. 2007; 365:513–522. [PubMed: 17067636]
- Parker ML, Ralston EJ, Eiserling FA. Bacteriophage SPO1 structure and morphogenesis. II. Head structure and DNA size. *J Virol*. 1983; 46:250–259. [PubMed: 6402606]
- Prasad BVV, Prevelige PE Jr, Marieta E, Chen RO, Thomas D, King J, Chiu W. Three-dimensional transformation of capsids associated with genome packaging in a bacterial virus. *J Mol Biol*. 1993; 231:65–74. [PubMed: 8496966]
- Prevelige PE Jr, Thomas D, King J. Scaffolding protein regulates the polymerization of P22 coat subunits into icosahedral shells *in vitro*. *J Mol Biol*. 1988; 202:743–757. [PubMed: 3262767]
- Prevelige PE Jr, Thomas D, King J. Nucleation and growth phases in the polymerization of coat and scaffolding subunits into icosahedral procapsid shells. *Biophys J*. 1993; 64:824–835. [PubMed: 8471727]
- Serwer P, Pichler ME. Electrophoresis of bacteriophage T7 and T7 capsids in agarose gels. *J Virol*. 1978; 28:917–928. [PubMed: 731798]
- Sideraki V, Huang W, Palzkill T, Gilbert HF. A secondary drug resistance mutation of TEM-1 beta-lactamase that suppresses misfolding and aggregation. *Proc Natl Acad Sci USA*. 2001; 98:283–288. [PubMed: 11114163]
- Steven AC, Couture E, Aebi U, Showe M. Structure of T4 polyheads. II. A pathway of polyhead transformation as a model for T4 capsid maturation. *J Mol Biol*. 1976; 106:187–221. [PubMed: 972397]
- Steven AC, Trus BL, Booy FP, Cheng N, Zlotnick A, Caston JR, Conway JF. The making and breaking of symmetry in the virus capsid assembly: glimpses of capsid biology from cryoelectron microscopy. *FASEB J*. 1997; 11:733–742. [PubMed: 9271358]
- Steven AC, Heymann JB, Cheng N, Trus BL, Conway JF. Virus maturation: dynamics and mechanism of a stabilizing structural transition that leads to infectivity. *Curr Opin Struct Biol*. 2005; 15:227–236. [PubMed: 15837183]
- Strauss H, King J. Steps in the stabilization of newly packaged DNA during phage P22 morphogenesis. *J Mol Biol*. 1984; 172:523–543. [PubMed: 6363718]
- Teschke CM. Aggregation and assembly of phage P22 temperature-sensitive coat protein mutants *in vitro* mimic the *in vivo* phenotype. *Biochemistry*. 1999; 38:2873–2881. [PubMed: 10074339]
- Teschke CM, Fong DG. Interactions between coat and scaffolding proteins of phage P22 are altered *in vitro* by amino acid substitutions in coat protein that cause a cold-sensitive phenotype. *Biochemistry*. 1996; 35:14831–14840. [PubMed: 8942646]
- Teschke CM, King J. Folding of the phage P22 coat protein *in vitro*. *Biochemistry*. 1993; 32:10839–10847. [PubMed: 8399234]
- Teschke CM, King J. *In vitro* folding of phage P22 coat protein with amino acid substitutions that confer *in vivo* temperature sensitivity. *Biochemistry*. 1995; 34:6815–6826. [PubMed: 7756313]

- Teschke CM, McGough A, Thuman-Commike PA. Penton release from P22 heat-expanded capsids suggests importance of stabilizing penton–hexon interactions during capsid maturation. *Biophys J*. 2003; 84:2585–2592. [PubMed: 12668466]
- Thomas PJ, Qu BH, Pedersen PL. Defective protein folding as a basis of human disease. *TIBS*. 1995; 20:456–459. [PubMed: 8578588]
- Thuman-Commike PA, Greene B, Jokana J, Prasad BVV, King J, Prevelige PE Jr, Chiu W. Three-dimensional structure of scaffolding-containing phage P22 procapsids by electron cryo-microscopy. *J Mol Biol*. 1996; 260:85–98. [PubMed: 8676394]
- Tuma R, Prevelige PE Jr, Thomas GJ Jr. Mechanism of capsid maturation in a double-stranded DNA virus. *Proc Natl Acad Sci USA*. 1998; 95:9885–9890. [PubMed: 9707570]
- Winston R, Botstein D, Miller JH. Characterization of amber and ochre suppressors in *Salmonella typhimurium*. *J Bacteriology*. 1979; 137:433–439.

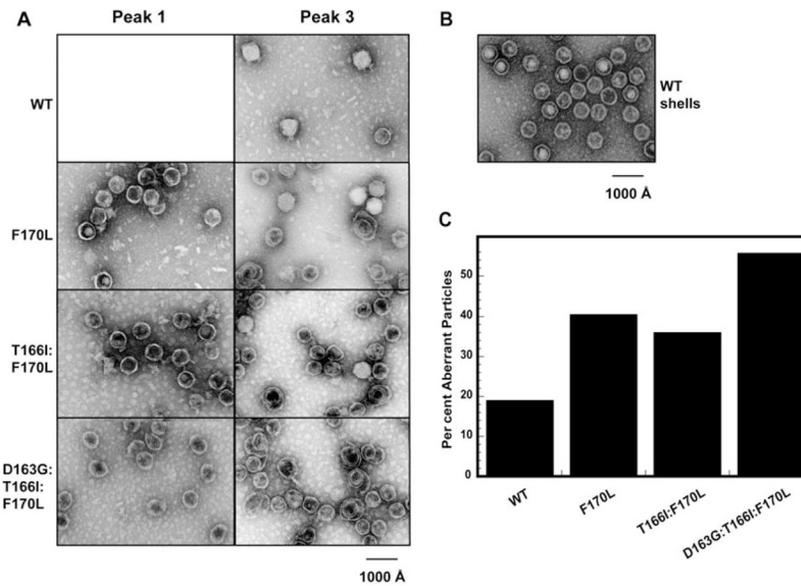


**Fig. 1.** Phage with multiple *su* substitutions demonstrate a *cs* phenotype. Phage that contained an amber suppressor in gene 5, which codes for coat protein, were complemented with coat protein expressed from a plasmid in DB7136 cells at temperatures ranging from 16°C to 41°C, as described in *Experimental procedures*. The relative titre is the titre of phage produced at each experimental temperature and condition divided by the titre of the phage at the permissive temperature and condition (30°C).



**Fig. 2.**

Expression of the *su* coat proteins in phage-infected cells results in an altered sucrose gradient profile. Cell lysates from phage infections conducted at 16°C with 5<sup>-</sup> amber phage complemented with WT, single-, double- and triple-*su*-substituted coat protein expressed from a plasmid were applied to 5–20% sucrose gradients. The sucrose gradient profile based on the intensity of coat protein in each fraction is shown for each protein type. The content of the peaks is described in the *Results* section.

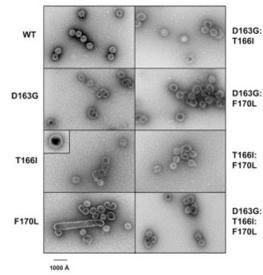


**Fig. 3.**

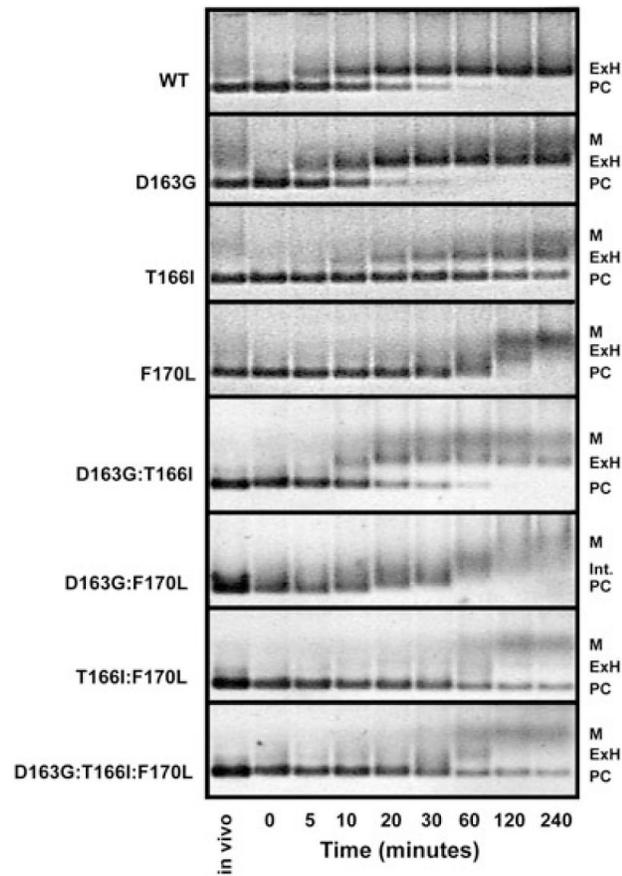
*Su* coat proteins result in empty heads and aberrant structures.

A,B. Micrographs of negatively stained samples from the sucrose gradients shown in Fig. 2. A shows representative micrographs of samples of peaks 1 and 3. For the WT coat protein sample, there was no detectable peak 1; therefore, the sample shown is from a sucrose gradient of purified WT empty shells, shown as a control in B.

C. The quantification of representative samples from several micrographs of peak 3 for some variants. The per cent aberrant structures was determined by dividing the number of empty heads, incorrectly sized particles, and any spirals by the total number of particles observed for each sample.

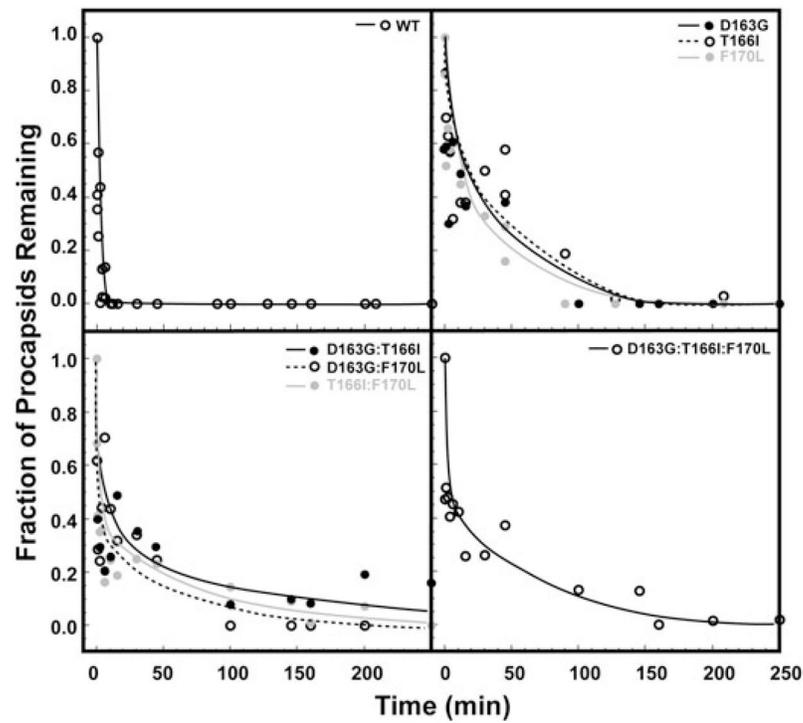


**Fig. 4.** Spirals and polyhead structures are formed during *in vitro* assembly reactions of the *su* coat proteins. Representative electron micrographs of negatively stained samples of *in vitro* assembly reactions from WT and *su* coat proteins are shown. The insert in the T166I panel corresponds to a procapsid-like particle with a larger diameter than normal, and is found in assembly reactions composed of D163G and T166I, but at a much lower frequency than the other aberrant structures. The insert is at the same magnification.



**Fig. 5.**

Procapsids formed from coat protein containing double and triple combinations of the *su* substitutions require longer incubation time to expand at low pH compared with procapsids formed from WT coat protein. Purified procapsids at  $1 \text{ mg ml}^{-1}$  were incubated in 20 mM citric acid at pH 3.0, at  $37^\circ\text{C}$  for 240 min; samples were taken with time, neutralized to pH 7.6 by addition of 1 M unbuffered Tris to stop expansion, and run on a 1.2% agarose gel at 17 V for 24 h at  $4^\circ\text{C}$ . The gels were Coomassie stained. Representative gels are shown that visualize the transition between procapsids and expanded structures for the coat protein variants. M, monomers; ExH, expanded heads; Int., intermediates, PC, procapsids.



**Fig. 6.** Procapsids formed from coat protein with double and triple combinations of the *su* substitutions are more resistant to the maturation reaction caused by heat than WT procapsids. Purified procapsids at  $1 \text{ mg ml}^{-1}$  were incubated in 20 mM sodium phosphate buffer at pH 7.6, at  $71^\circ\text{C}$  for 250 min; and samples were taken with time and placed on ice to stop expansion. Samples were then run on a 1.2% agarose gel at 17 V for 24 h at  $4^\circ\text{C}$  and Coomassie stained. The intensity of each procapsid band was determined by densitometry using a Kodak EDAS. The fraction of coat protein remaining was determined by dividing the procapsid band intensity at each experimental time by the intensity of the initial sample.

**Table 1**

Primers used for Quikchange.

Plasmid		
pD163G <sup>a</sup>	Forward primer	CCGCGAACTTAACCGCGGCATGGGG
	Reverse primer	CCCCATGCCGCGGTTAAGTTCGCGG
pT166I <sup>a</sup>	Forward primer	CCGCGAACTTAACCGCGCATGGGGATATCG
	Reverse primer	CGATATCCCCATGTCGCGGTTAAGTTCGCGG
pF170L <sup>a</sup>	Forward primer	TGGGGACATCGTACTTCCTCAACCCTCAGG
	Reverse primer	CCTGAGGGTTGAGGAAGTACGATGTCCCA
pD163G:T166I <sup>b</sup>	Forward primer	CCGCGAACTTAACCGCGGCATGGGGATATCG
	Reverse primer	CGATATCCCCATGCCGCGGTTAAGTTCGCGG
pD163G:F170L <sup>b</sup>	Forward primer	CCGCGGCATGGGGACATCGTACTTCCTCAACCC
	Reverse primer	GGGTTGAGGAAGTACGATGTCCCATGCCGCGG
pT166I:F170L <sup>c</sup>	Forward primer	CCGCGCATGGGGATATCGTACTTCCTCAACCCTCAG
	Reverse primer	CTGAGGGTTGAGGAAGTACGATATCCCATGTCGCGG
pD163G:T166I:F170L <sup>d</sup>	Forward primer	CCGCGAACTTAACCGCGGCATGGGGATATCG
	Reverse primer	CGATATCCCCATGCCGCGGTTAAGTTCGCGG

All primers are written 5'–3'.

The nucleotide in bold matched the *su* substitution.

<sup>a</sup> pWT was the template.

<sup>b</sup> pD163G was the template.

<sup>c</sup> pT166I was the template.

<sup>d</sup> pT166I:F170L was the template.